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γ-Glutamyl transpeptidase GGT4 initiates vacuolar degradation of glutathione *S*-conjugates in *Arabidopsis*

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Abstract **The xenobiotic monochlorobimane is conjugated to glutathione in the cytosol of *Arabidopsis thaliana*, transported to the vacuole, and hydrolyzed to cysteine *S*-bimane [Grzam, A., Tennstedt, P., Clemens, S., Hell, R. and Meyer, A.J. (2006) Vacuolar sequestration of glutathione *S*-conjugates outcompetes a possible degradation of the glutathione moiety by phytochelatin synthase. FEBS Lett. 580, 6384–6390]. The work here identifies γ-glutamyl transpeptidase 4 (At4g29210, GGT4) as the first step of vacuolar degradation of glutathione conjugates. Hydrolysis of glutathione *S*-bimane is blocked in *ggt4* null mutants of *A. thaliana*. Accumulation of glutathione *S*-bimane in mutants and in wild-type plants treated with the high affinity GGT inhibitor acivicin shows that GGT4 is required to initiate the two step hydrolysis sequence. GGT4:green fluorescent protein fusions were used to demonstrate that GGT4 is localized in the lumen of the vacuole.**

Keywords: γ-Glutamyl transpeptidase; Glutathione-conjugate degradation; Monochlorobimane; Vacuolar sequestration

1. Introduction

Plants take up many toxic xenobiotics from their growth environment with relative indiscrimination but are able to detoxify or sequester these compounds by a number of pathways [1]. Detoxification of many electrophilic xenobiotics begins by reaction with reduced glutathione (GSH). Xenobiotics are covalently bound to the sulfhydryl group of GSH in a reaction catalyzed by glutathione *S*-transferases (GSTs) [2]. Following the conjugation in the cytosol, the glutathione *S*-conjugates undergo further metabolism which results in transient accumulation of degradation products [3,4]. A vacuolar carboxypeptidase activity that cleaved the Gly residue as the initial reaction with the concomitant accumulation of γ-GluCys-conjugates was shown in barley vacuoles [5]. In contrast to barley, *Arabidopsis thaliana* plants challenged with xenobiotics accumulate mainly Cys *S*-conjugates and only minute amounts of intermediate degradation products [6]. Accumulation of Cys *S*-conjugates implies that the two initial steps in glutathione *S*-conjugate metabolism involve cleavage of Glu and Gly from the GSH-moiety of the conjugates. The absence of intermediates showed that the first degradation step limits the overall reaction but provided no information as to whether the Glu or the Gly residue are cleaved first [6]. Recent reports implicate two different enzymes, phytochelatin synthase (PCS) and γ-glutamyl transpeptidase (GGT), as catalysts for the initial step of glutathione *S*-conjugate degradation in *Arabidopsis* [7,8]. Cytosolic PCS is able to initiate glutathione *S*-conjugate metabolism by removal of the Gly residue [7,9]. It needs to be considered that PCS is almost inactive unless plants are exposed to heavy metal contamination. Recent reports showed that in the absence of heavy metals vacuolar sequestration of glutathione *S*-conjugates occurs much more rapidly than hydrolysis of the glutathione moiety by PCS in the cytosol [6,10].

Significant activity towards glutathione *S*-conjugates was also shown for a vacuolar GGT [8]. Given that glutathione *S*-conjugates are rapidly sequestered to the vacuole, degradation of at least a significant proportion within the vacuole seems more plausible than degradation in the cytosol as proposed by Blum et al. [7]. GGTs are the only enzymes known to hydrolyze the unique amide bond linking the γ-carboxylic acid of Glu to Cys in GSH. Several GGT isoforms have been purified from plant species including tomato, onion, and radish [11–13]. Like most GGTs from animal tissues, the GGTs from these plants exhibited broad substrate specificity and were able to hydrolyze GSH and several glutathione *S*-conjugates [14,15]. The bulk of the GGT activity in these plants was localized in a pellet fraction and extractable only with high molarity NaCl, suggesting ionic association possibly with the cell wall. However, Nakano and colleagues showed that a less abundant soluble GGT from radish is localized to the vacuole and is able to degrade glutathione *S*-bimane (GSB) [13,16]. In the yeast *Saccharomyces cerevisiae*, GGT is a membrane bound vacuolar protein [17].

In *Arabidopsis* four GGT genes have been identified. According to gene symbols registered with the *Arabidopsis* information resource (TAIR) these genes are named GGT1 (At4g39640), GGT2 (At4g39650), GGT3 (At1g69820), and GGT4 (At4g29210) [18,19]. GGT1, GGT2, and GGT3 exhibit between 80% and 90% identity among each other and have

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Abbreviations: CHX, cycloheximide; CLSM, confocal laser scanning microscopy; Cys-B, cysteine *S*-bimane; CysGly-B, cysteinylglycine-bimane; GGT, γ-glutamyl-transpeptidase; GSB, glutathione *S*-bimane; GSH, reduced glutathione; GST, glutathione *S*-transferase; LMWT, low molecular weight thiol; MBB, monobromobimane; MCB, monochlorobimane; PCS, phytochelatin synthetase

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highly conserved intron/exon structures. GGT4 is divergent from the other GGTs with homology of about 50% [18–20]. Heterologous expression of *At*GGT1 in tobacco and analysis of GGT1 knockouts indicated that GGT activity is localized outside the plasma membrane and likely associated ionically with the cell wall [18–20]. The other GGTs have not been characterized in detail and GGTs were not identified in recent surveys of the vacuolar proteome [21,22]. However, prediction of subcellular targeting of the four GGTs using the combination of algorithms assembled at the SubCellular Proteomic Database (SUBA) site (http://www.plantenergy.uwa.edu.au.application/suba/flatfile/) identified GGT4 as possibly targeted to the vacuole [23].

Fluorescent in situ labelling of GSH with monochlorobimane (MCB) offers the unsurpassed opportunity to trace the GSH-dependent detoxification pathway in living cells [10]. The initially non-fluorescent and membrane-permeable MCB is conjugated to GSH in a reaction catalyzed by GSTs leading to formation of fluorescent glutathione *S*-bimane (GSB). After vacuolar sequestration, the fluorescence is not affected by the first two hydrolysis reactions that lead to formation of cysteine *S*-bimane (Cys-B). The persistent fluorescence also allows extraction of MCB labelled thiols from tissues for direct analysis [6].

In situ labelling of metabolites with MCB in combination with reverse genetics were used to show that GGT4 is the vacuolar enzyme responsible for cleaving the γ-glutamyl residue from GSB as the initial glutathione *S*-conjugate degradation step following exposure of *Arabidopsis* to toxic levels of xenobiotics.

2. Materials and methods

2.1. Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh. (accessions Landsberg *erecta*, L*er* and Columbia, Col-0) and 2 mutant lines, lacking GGT4 (At4g29210) were used. The transposon insertion mutants designated *ggt4-1* and *ggt4-2* both in background L*er* were obtained from the EU Exon Trapping Consortium (Exotic-GT-5-57895) and Cold Spring Harbor Laboratory (CSHL-GT11203), respectively [24].

For experiments with leaf material, plants were sown on soil and grown for 8–10 weeks at 21 °C under short day conditions (9 h light; 15 h dark) in a controlled growth chamber. For in situ experiments with root material, plants were grown hydroponically. Seeds were sterilized and sown on growth medium containing 0.8% agar in 0.5 ml Eppendorf tubes with bottoms removed. These seed holders were placed on containers filled with 0.5× Hoagland medium and plants were grown for 10 weeks.

2.2. Analysis of mutant lines

For verification of the insertion site, genomic DNA was extracted from 20 d old plants grown at 24 °C with 16 h light; 8 h dark. PCR amplification was performed using an Extract N-Amp Kit (Sigma Chemical Co., St. Louis, MO) and primers specific for the transposon LB and a flanking GGT4 sequence. Primers used were: GGT4 specific FP, aatcggtggtggttctttcttgatt; GGT4 specific RP, tgttgagaaatcatccatctcgttg; and Exotic-GT-TPS-3′DS, acccgaccggatcgtatcggt. Amplified products were sequenced to verify the insertion site.

Homozygous plants of each line were selected on kanamycin and were also confirmed by PCR. Knockout of gene function was confirmed by RT-PCR. RNA was extracted from wild-type and mutants using TRIzol reagent (Invitrogen, Carlsbad, CA), first strand cDNA synthesized using Superscript II reverse transcriptase (Invitrogen), and products were amplified by PCR using Platinum Taq DNA Polymerase (Invitrogen) and the gene specific primers indicated above. Actin 2 (ACT 2) was used as an internal control.

2.3. DNA constructions and plant transformation

For transient expression of GGT4:GFP fusions, a short N-terminal fragments of 85 amino acids of AtGGT4 cDNA (RAFL15-32-I09; RIKEN Genomic Sciences Centre, Japan) were amplified and cloned with *Bam*HI and *Sal*I in front of eGFP in the vector pFF19 [25]. Transformation of onion epidermal cells and *Arabidopsis* leaves was done by particle bombardment. Gold particles were prepared according to the manufacturers protocol (Bio-Rad, München, Germany) and the particle suspension was spotted to macro carriers with 3–6 μg DNA per shot. Tissues were subjected to particle bombardment at 650 psi (onion) or 900 psi (*Arabidopsis*) (Biolistic PDS-1000-He; Bio-Rad). The bombarded tissue was kept in Petri dishes on wet filter paper for 24–72 h at room temperature in the dark.

2.4. Leaf infiltration for GSH labelling

For analysis of GSB and its degradation products, infiltration, incubation, harvesting and reverse-phase HPLC analysis of leaf samples was performed as described [6]. Where indicated, buffers for leaf infiltration and subsequent incubation were supplemented with 500 μM acivicin and 1.4 mM cycloheximide (CHX) as inhibitors for degradation and protein biosynthesis as indicated. For analysis of gene expression 100 mg leaf material was used. This material was infiltrated with either buffer (control) or with buffer containing 300–500 μM MCB or monobromobimane (MBB). All other conditions for infiltration, incubation and harvesting were the same as described above.

2.5. Root incubation for GSH labelling

Roots of 10 weeks old hydroponically grown plants were cut off and washed with infiltration buffer. After careful removal of adhering water 150–250 mg fresh root material was cut in pieces of 4–8 mm length and placed in fresh infiltration buffer. The root material was incubated in MCB solution for 15 min at RT in the dark. After washing twice with fresh incubation buffer, a sample of 50–100 mg was taken immediately after washing (time point 0), and two further samples were taken after extended incubation for 5 h and 24 h at RT in the dark. Extraction and analysis of thiol bimane conjugates was performed as described for leaves. Due to different labelling efficiencies and to avoid possible errors with determination of the fresh weight of small root samples the amounts of GSB and its degradation products are presented as % of all recovered bimane-labelled low molecular weight thiols (LMWT).

2.6. Confocal microscopy

Imaging of bimane conjugates formed in situ after labelling of GSH with 100 μM MCB was done as described earlier [6,10]. GFP fluorescence was observed with either a 25× multi-immersion lens (Plan-Neofluar, NA 0.8, Zeiss, Jena, Germany) or a 63× water-immersion lens (C-Apochromat, NA 1.2; Zeiss). Images were assembled in Photoshop (Adobe Systems).

3. Results

3.1. Identification and molecular characterization of ggt4 insertional mutants

To investigate the role of GGT4 in the metabolism of glutathione *S*-conjugates, two independent transposon insertion lines for this gene were identified. The GGT4 gene consists of a 3300 bp open reading frame, and the gene model, supported by cDNAs, shows 5 exons (Fig. 1A). Each knockout line contained a single locus transposon insertion in exon 3. The sequence of DNA spanning the insertion site in each mutant was determined to confirm the position of insertion and is shown in Fig. 1A. The lines were designated *ggt4-1* and *ggt4-2*.

Neither *ggt4-1* nor *ggt4-2* differed from wild-type plants at any stage during development when grown under several different growth conditions. RT-PCR analysis with mRNA from 20-day-old leaves established that both insertion lines are indeed null mutants with respect to GGT4 expression

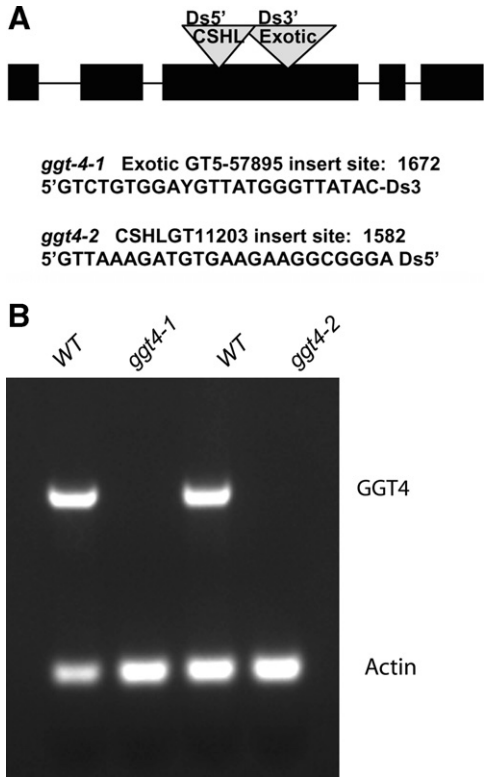

Fig. 1. Molecular characterization of *ggt4* insertional knockouts. (A) Genomic structure of GGT4 and localization of transposon insertions. Line numbers, insertion sites, and sequence at the insertion sites are shown below the gene model. (B) RT-PCR analysis shows GGT4 and actin transcript accumulation in *ggt4-1*, and *ggt4-2* and the corresponding wild-type segregants.

(Fig. 1B). Using GGT4-specific primers, expression was demonstrated in the wild-type segregant from both lines, while *ggt4-1* and *ggt4-2* yielded no product.

3.2. GGT4 is involved in degradation of glutathione S-conjugates

In light of the predicted localization of GGT4, we focused on the degradation of glutathione *S*-conjugates in *ggt4* deletion mutants. For wild-type plants of the ecotype Col-0, we recently showed fast degradation of GSB after infiltration of leaf tissue with MCB [6]. Given that all available *ggt4* mutants are in ecotype L*er* background, we here also established the time course for MCB degradation for L*er* as a wild-type control. Under the conditions used for MCB treatment a major fraction of the cellular GSH pool was labelled with bimane similar to the situation we have previously established in Col-0 plants. Immediately after infiltration with MCB, i.e. time point zero of the time course, more than 90% of the bimane was present as GSB and only residual amounts as conjugates of other LMWTs (Fig. 2A and B). In wild-type leaves 24 h after exposure to MCB, about 60% of the label was present as Cys-B indicating significant degradation of GSH conjugates during this period (Fig. 2B). The possible intermediates, γ-GluCys or CysGly, did not accumulate to significant extent (Supplementary Fig. S1). Degradation of GSB was blocked in *ggt4-1* mutant plants. Cys-B accumulated only to about 10% of all LMWTs in 24 h (Fig. 2B) and 90% of bimane was still present as GSB, indicating that the first step of degradation was blocked.

Exposure of intact wild-type roots to MCB resulted in similar degradation of GSB when compared to leaves (Fig. 2C). The only obvious difference was accumulation of cysteinylglycine-bimane (CysGly-B) in roots to a level of about 12% during the 24 h incubation period, whereas no accumulation was

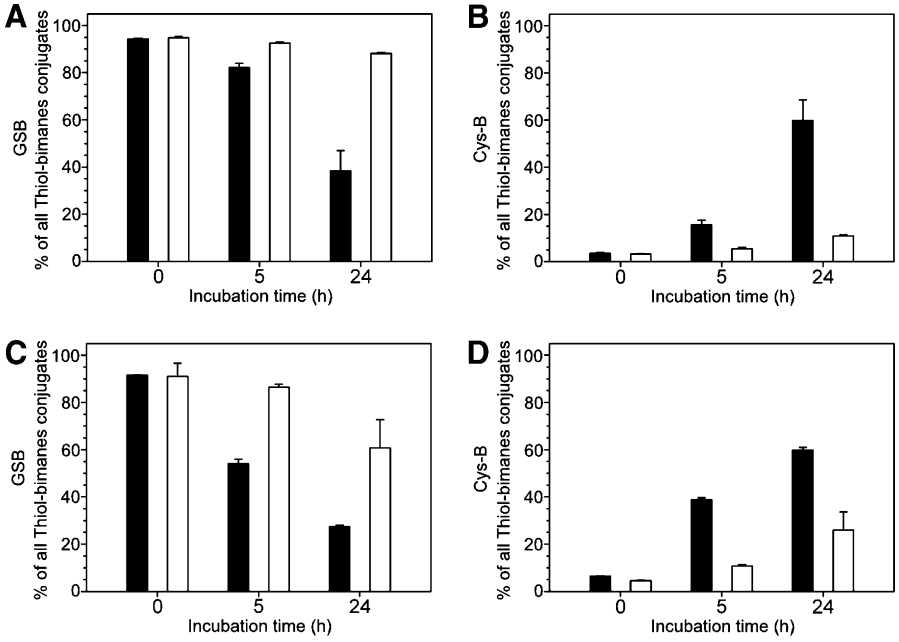

Fig. 2. Degradation of GSB in *Arabidopsis* requires GGT4 activity. Leaves and roots were infiltrated for 15 min with 500 μM MCB. Samples were harvested immediately (time point 0) or after incubation for 5 or 24 h without MCB. Thiol-bimane conjugates were extracted and analyzed by HPLC. Wild-type is shown in black and *ggt4-1* in white. (A) Percent of thiol-bimane present as GSB in leaves. (B) Percent of thiol-bimane present as Cys-B in leaves. (C) Percent of thiol-bimane present as GSB in roots. (D) Percent of thiol-bimane present as Cys-B in roots. All values are means ± SD; $n = 5$.

observed in leaves (Fig. S1). In the roots of *ggt4-1*, however, 40% of the GSB was metabolized within 24 h and of that 30% of the label was found as Cys-B (Fig. 2D). Since no differences were observed between the two mutant lines, experimental results are shown in Fig. 2 and subsequent figures only for *ggt4-1*.

3.3. Glutathione S-conjugate degradation and GGT4 are located in the vacuole

Glutathione *S*-conjugates formed in the cytosol of plant cells are generally sequestered to the vacuole for further metabolism. MCB-labelling of cytosolic GSH exploits the GSH-dependent detoxification pathway. Observation of living cells shows rapid transfer of the GSB to the vacuole [10]. To test whether knocking out glutathione *S*-conjugate hydrolysis altered sequestering of the conjugate to the vacuole, we imaged mature leaves of wild-type and *ggt4-1* 40–60 min after infiltration with MCB. The conjugate was almost completely transferred within 30 min in both wild-type and *ggt4-1*, leaving the GSH-depleted cytosol negatively contrasted (Fig. 3; videos of serial optical sections along *z*-axis as supplements).

The results in Fig. 2 strongly suggest that GGT4 is involved in GSB degradation. Since bioinformatics tools predicted that the enzyme is located in the vacuole, experiments were designed to verify the prediction. GFP was C-terminally fused to full-length and truncated versions of GGT4 and transiently expressed in different plant species. Tagging of full length and several truncated GGT4 protein resulted in only low expression, which did not allow drawing conclusive results about the localization (not shown). According to the ARAMEMNON database [26] GGT4 is a transmembrane protein with three predicted transmembrane domains. Based on the predicted topology GFP was then fused to the N-terminal 85 amino acids of GGT4, which include the first transmembrane domain. This truncated $GGT4_{1-85}$:GFP fusion protein resulted in high expression and clear vacuolar labelling two days after transient transformation of onion epidermal cells and also *Arabidopsis* epidermal cells (Fig. 4B and D). In case of *Arabidopsis*, some fluorescence was also observed in compartments along the secretory pathway (Fig. 4D). GFP expressed on its own without any target signal always remained in the cytosol (Fig. 4A and C).

3.4. Acivicin inhibits GGT4

Acivicin is a high affinity in vitro inhibitor of mammalian and plant GGTs [13,27]. Infiltration of 500 μM acivicin together with MCB completely blocked the GSB-degrading activity in leaves (Fig. 5A and B). In roots GSB degradation was not fully abolished in *ggt4* mutants (Fig. 2C and D). The residual GSB degradation observed in *ggt4* mutants was however further reduced by acivicin and also reflected by diminished Cys-B formation (Fig. 5C and D). This suggests that the residual GSB degradation results at least in part from other GGTs.

3.5. Continuous GSB degradation depends on stable expression of GGT4

Fig. 1B illustrated the expression level in 20 d rosette leaves. In fact, our RT-PCR results (not shown) and query of public microarrays using GENEVESTIGATOR [28] or the *Arabidopsis* eFP Browser (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi) show that the GGT4 transcript is present in most tissues of non-stressed wild-type plants. To test whether sustained degradation of GSB under conditions of high doses of xenobiotics required new protein biosynthesis, *Arabidopsis* leaves were simultaneously infiltrated with 500 μM MCB and 1.4 mM CHX as an inhibitor of translation. CHX significantly slowed down the degradation of GSB (Fig. 6). During the first 5 h of in situ labelling with MCB, control leaves and CHX treated leaves were able to degrade about 15% of the GSB. After 5 h, the control leaves further degraded GSB at an almost linear rate leaving only 30% after 24 h, while degradation in CHX treated leaves came to a halt with about 80% of GSB remaining (Fig. 6A). The concomitant accumulation of Cys-B reflected the degradation of GSB without accumulation of intermediates in either case (Fig. 6B). Cell membrane integrity was maintained during CHX treatment indicating that cells were alive even after 24 h (not shown). Concomitant analysis of expression by RT-PCR did not reveal significant induction of GGT4 after infiltration of leaves with 300–500 μM MCB or MBB (Supplementary Fig. S2).

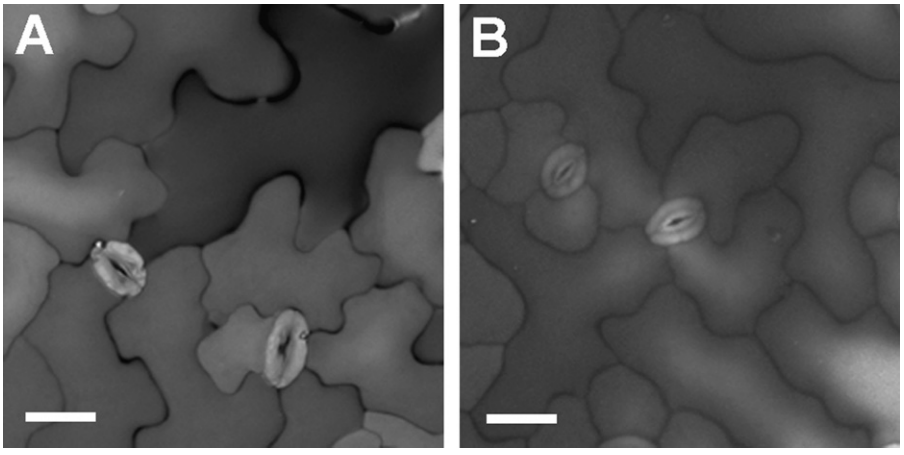

Fig. 3. Vacuolar sequestration of GSB is not altered in *ggt4-1*. Leaves were infiltrated with 500 μM MCB for 15 min and then incubated without MCB for 40–60 min. Serial optical sections taken by CLSM with excitation at 405 nm are presented as projections along the *z*-axis. (A) wild-type; (B) *ggt4-1*. Scale bars = 20 μm.

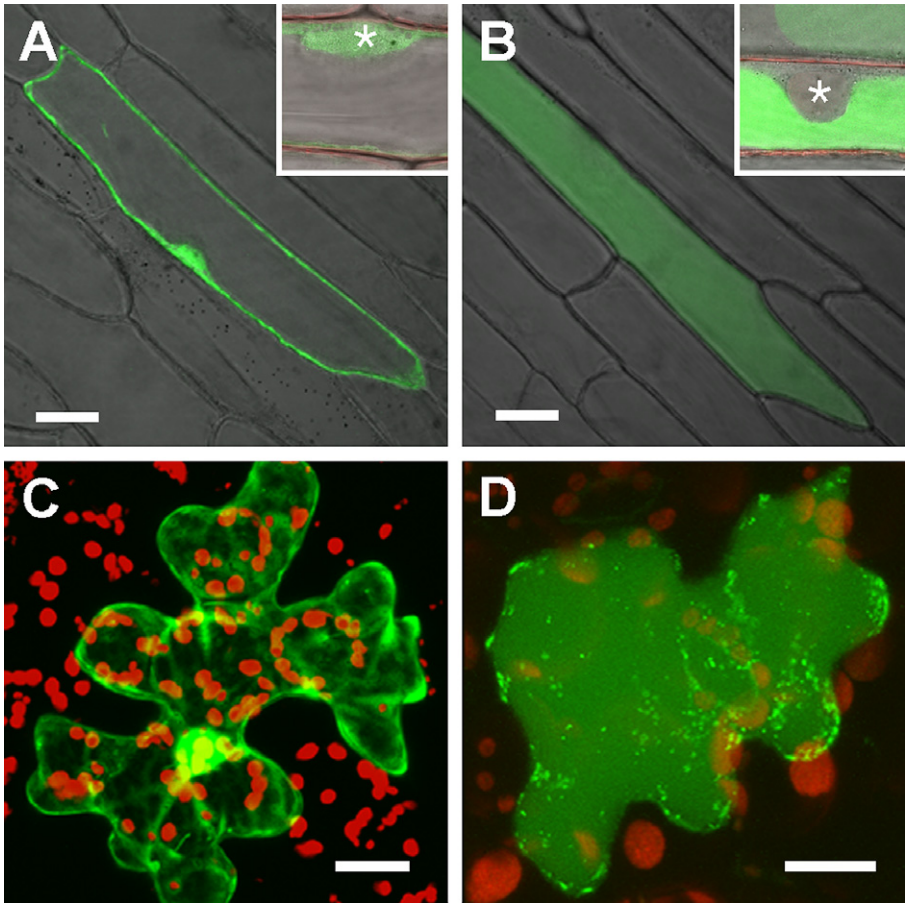

Fig. 4. GGT4:GFP fusion proteins are targeted to the secretory pathway and the vacuole. GFP alone (A and C) and $GGT4_{1-85}$: GFP fusions (B and D) were transiently expressed after particle bombardment in epidermal cells of onion and *Arabidopsis*. Images of were taken by CLSM two days after transfection. Onion epidermal cells (A and B). Insets show GFP fluorescence in the nuclear regions (*) at higher magnification. Scale bars = 50 μm. *Arabidopsis* leaf epidermal cell transformed with GFP (C) or with a $GGT4_{1-85}$:GFP fusion (D). Images are projections from serial optical sections along the *z*-axis. Scale bars = 20 μm and 10 μm, respectively.

4. Discussion

Formation of glutathione *S*-conjugates is an important detoxification mechanism to counter the phytotoxicity of a wide variety of xenobiotics and perhaps many endogenous compounds. After conjugate formation within the cytosol, the glutathione moiety effectively acts as a tag for immediate sequestration to the vacuole [10,29,30]. Inside the vacuole, conjugates undergo several modifications. These reactions typically start with cleavage of Glu and Gly from the glutathione moiety leading to accumulation of cysteine-tagged conjugates [6]. In the few plant species examined to date, γ-GluCys-conjugates were reported as the first intermediate in glutathione *S*-conjugate metabolism in some species while in others CysGly-conjugates were detected [3,4]. However, comparison of the *ggt4* plants with wild-type plants unequivocally shows that the initial degradation step, at least in *Arabidopsis*, is catalyzed by GGT4. Almost complete blockage of GSB degradation in leaves of *ggt4* plants showed that GGT4-catalyzed degradation is the only viable pathway for degradation. In roots of *ggt4* plants, about 30% of the bimane-labelled thiols were present as Cys-B after 24 h suggesting that another enzyme might work in parallel. This result corroborates the recently published observations by Ohkama-Ohtsu and colleagues on the same enzyme [8].

In theory, sequential hydrolysis of the two amide bounds of the glutathione tag could be initiated from either end. A carboxypeptidase activity, able to cleave Gly to produce the intermediate γ-GluCys-conjugate, has been detected in barley vacuoles [5], but this activity appears to be absent from *Arabidopsis* leaves. In tomato, GGTs are able to hydrolyze both GSH and γ-GluCys [11]. However, the absence of significant intermediates in the *ggt4* mutant indicates that in *Arabidopsis* degradation of glutathione *S*-conjugates strictly occurs by the ordered removal of Glu first and Gly second. The presence of a carboxypeptidase in roots can not be ruled out at this point.

The identity of GGT4 as the enzyme responsible for vacuolar degradation is further supported by different molecular and biochemical approaches. Simultaneous incubation of wild-type leaves and roots with MCB and the high affinity GGT inhibitor acivicin [13,27], did not result in intermediates indicating that hydrolysis could not be initiated from the C-terminus of the glutathione moiety. The sequential order of hydrolysis of glutathione *S*-conjugates in *Arabidopsis* is thus identical to the order of reactions observed for both GSH and glutathione *S*-conjugates in animals where removal of the γ-glutamyl residue is also the first and rate-limiting step [31].

There are, however, distinct differences between animal and plant xenobiotic detoxification. For one, animals export gluta-

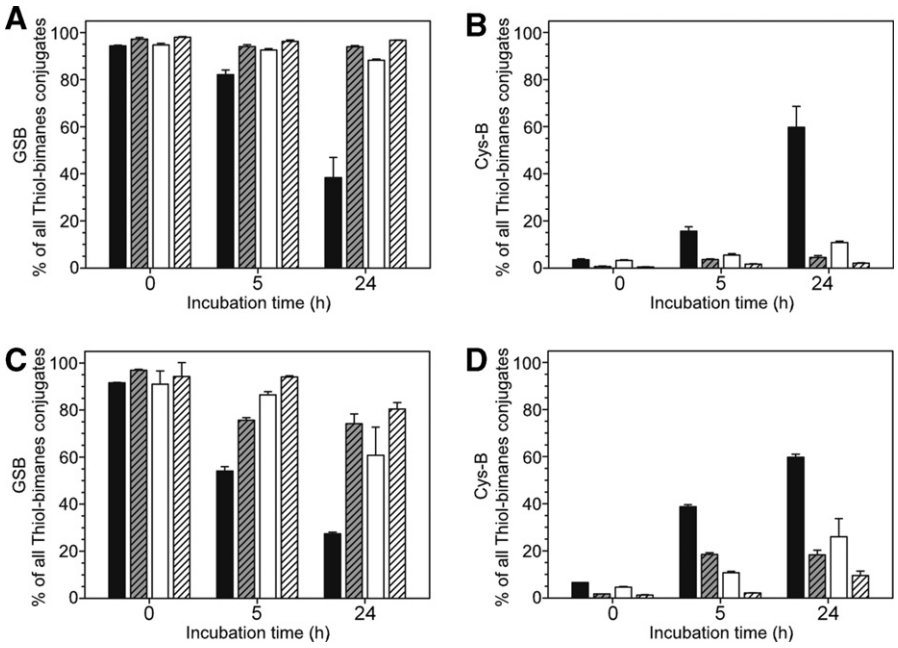

Fig. 5. Degradation of GSB requires GGT4 activity and is inhibited by acivicin. Leaves (A and B) and roots (C and D) of wild-type (black) or *ggt4-1* (white) were infiltrated with 500 μM MCB in the absence or presence of 500 μM acivicin (striped bars). After 15 min infiltration, samples were harvested immediately (time point 0) or after incubation for 5 or 24 h in buffer without MCB. Thiol-bimane conjugates were extracted and analyzed by HPLC. (A) GSB in leaves; (B) Cys-B in leaves; (C) GSB in roots; (D) Cys-B in roots. Values are shown as means ± S.D. of 6–9 independent measurements.

thione *S*-conjugates from the cell for further metabolism whereas plants sequester the conjugates to the vacuole for further metabolism. This sequestration process can be monitored in vivo with fluorescent substrates for GST-catalysed conjugation reactions [10,32].

Second, in mice, the GGT and CysGly peptidase responsible for conjugate hydrolysis are anchored on the external surface of the cell membrane. In contrast, localization of *Arabidopsis* GGT4 in the vacuole is supported by the observation that transiently expressed GGT4:GFP fusions are targeted to the vacuole. This is in agreement with predictive algorithms at the SUBA database that localize GGT4 to the vacuole [23]. The consensus of ARAMEMNON predictors [26] is that GGT4 has three membrane spanning helices and that the largest part of the protein would be cytoplasmic, which would be unlikely given that the substrate GSB clearly is sequestered to the vacuole lumen. However, experimental results are in agreement with some predictive algorithms, including TMHMM (http://www/cbs.dtu.dk/services/TMHMM-2.0) that identify a single N-terminal domain that might either be inserted in the membrane or cleaved. Fluorescence of the GGT4:GFP construct appears to be localized in the lumen of the vacuole rather than bound to the tonoplast membrane. While the vacuolar localization of the GGT4:GFP fusion per se is clear, it might not reliably reflect whether the protein is soluble or membrane associated, since only the N-terminal 85 amino acids of GGT4 were utilized.

Both, soluble and "bound" GGTs have been identified in *Arabidopsis*, onion, tomato, and radish [8,11–13,16,18–20]. The majority of GGT activity in all examined species is "bound" possibly via an ionic association with the cell wall. In all species, GGT activity was released with high molarity NaCl, and in some cases it was shown to be lost upon protoplast formation. In *Arabidopsis*, GGT1 was shown to be the

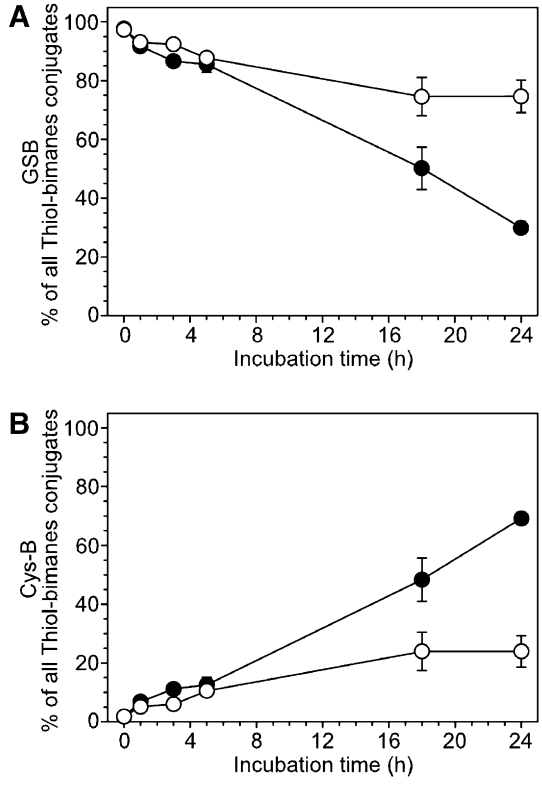

Fig. 6. Sustained degradation of GSB in *Arabidopsis* requires protein synthesis. Leaf pieces were infiltrated with 500 μM MCB in the absence (●) and in the presence (○) of 1.4 mM cycloheximide. After 15 min infiltration, samples were harvested immediately (time point 0) or after further incubation for 5 or 24 h in buffer without MCB and with (○) or without (●) cycloheximide. Thiol-bimane conjugates were extracted and analysed by HPLC. GSB (A) and Cys-B (B) are shown as means ± S.D.; $n = 3$.

bound activity, and it was found to be localized to the apoplast space [18–20]. However, in radish a soluble GGT was reported to be localized to the vacuole [16].

Analysis of GGT4 expression using microarray data at public databases and GENEVESTIGATOR tools [28] showed that GGT4 is expressed at a low level in nearly all *Arabidopsis* tissues. Low expression was also observed with GGT4::GUS fusions using the native GGT4 promoter and the full length ORF [19]. A low endogenous level of GGT4 in the vacuole would explain the failure to identify GGT4 in the vacuolar *Arabidopsis* proteome [21]. After treatment with 2 μM, 2,4-dichlorophenoxyacetic acid for three days a 1.6-fold increase in GGT4 expression was reported by Ohkama-Ohtsu et al. [8], but it is not clear whether this was due to gradual depletion of GSH or some other side effect of the herbicide. Analysis of publically available microarray datasets using GENEVESTIGATOR [28] or *Arabidopsis* eFP Browser (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb) showed induction of GGT4 transcript of 2-fold or less in most cases after infection with a number of bacterial and fungal pathogens, by wounding, and by several chemical treatments including ozone and methyl jasmonate. The more severe but highly specific depletion of GSH by exposure to high concentrations of MCB or MBB, in contrast, did not trigger increased gene expression. This result strongly dismisses the possibility that GGT4 expression is dependent on the cytosolic GSH level. The observed almost linear rate of GSB degradation over several hours suggests that the steady state expression is sufficient for degradation. Degradation was first reduced and ultimately halted by inhibiting protein biosynthesis with CHX. Together with lack of GGT4 induction this result suggests that continuous expression is apparently necessary to cover loss of GGT4 due to normal protein turnover.

All results presented in this work are consistent with GGT4 being the enzyme responsible for initiating vacuolar degradation of glutathione *S*-conjugates through removal of the Glu residue. The contrast to the recent suggestion that cytosolic PCS is the key enzyme catalyzing the initial step of degradation by removing the Gly moiety [7] cannot be finally resolved at this stage and may depend on environmental and experimental conditions. Despite a very similar experimental approach based on in situ labelling of GSH with MCB there are some significant differences. The experiments presented in this work were all conducted with 15 min incubation of tissue with high concentrations of MCB. Blum and colleagues [7] in contrast used only 5 μM MCB and an incubation time of 4 h. It might be possible that under these conditions the concentration of GSB in the cytosol is not high enough for efficient vacuolar sequestration of GSB and thus GSB might be accessible to PCS in this case. In our earlier work it was shown that under conditions of severe exposure to xenobiotics vacuolar sequestration of GSB is significantly faster than the possible degradation by PCS in the cytosol [6]. It is now clear that under these circumstances GGT4 is the key enzyme for initiating the degradation of glutathione *S*-conjugates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.05.071.

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